Importance of Host Plant Species, *Neotyphodium* Endophyte Isolate, and Alkaloids on Feeding by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) Larvae

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ABSTRACT Three grass host species—tall fescue, *Festuca arundinacea* Schreber; meadow fescue, *Festuca pratensis* Hudson; and perennial ryegrass, *Lolium perenne* L.—each infected with a number of different *Neotyphodium* endophyte isolates, were investigated for their effects on fall armyworm, *Spodoptera frugiperda* (J.E. Smith). Alkaloid profiles varied among associations. Choice and no-choice tests comparing feeding and early development of *S. frugiperda* larvae on endophyte-infected and endophyte-free leaf blade material were performed. Endophyte-mediated resistance to *S. frugiperda* was greatest in meadow fescue and weakest in tall fescue. Some endophyte isolates, particularly in perennial ryegrass and meadow fescue, had a major effect on feeding and development of *S. frugiperda*, whereas others had no effect or were only weakly efficacious. In tall fescue, some associations deterred *S. frugiperda* from feeding in choice tests but had no effect on development, whereas larvae reared on other associations weighed significantly more than control larvae fed endophyte-free grass. It was concluded that the deleterious consequences of endophyte infection were easily masked by other factors in tall fescue. Relative leaf age had no effect on feeding preferences in the three host species. Chemical analysis of herbage from the plants used, and results from a no-choice study using spiked artificial diets, failed to individually implicate any of the major known alkaloids (peramine, lolitrem B, ergovaline, and lolines) in the observed effects on *S. frugiperda*. Hypotheses explaining these observations, and their impact on creating desirable grass–endophyte associations for use in pastures, are discussed.

KEY WORDS insect resistance, alkaloid, armyworm, fescue, ryegrass

Symptomless endophytic fungi of the genus *Neotyphodium* (Ascomycota: Clavicipitaceae), which may infect important forage grass species, have come to prominence because their presence has been linked to health problems in livestock (Bacon et al. 1977, Hoveland et al. 1980, Fletcher and Harvey 1981) and to several beneficial host plant effects, including resistance to insect pests such as the weevil *Listronotus bonariensis* (Kuschel) (Prestidge et al. 1982). In most areas of New Zealand, the insect resistance component is so important that persistence and productivity of the predominant graminaceous pasture species, perennial ryegrass, *Lolium perenne* L., is very poor unless a large proportion of the plants are infected with *Neotyphodium lolii* (Latch, Christensen, and Samuels) Glenn, Bacon, and Hanlin (Popay et al. 1999). In the southeastern United States, the most important forage species is tall fescue, *Festuca arundinacea* Schreber, the performance of which is greatly improved if plants are infected with *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon, and Hanlin (Read and Camp 1986). Insect resistance mediated by *N. coenophialum* is likely to be a significant factor in this improved performance, although an increased tolerance to drought is probably more important (Bouton et al. 1993).

Since these discoveries, a primary aim of endophyte research has been to develop associations between important pasture species such as tall fescue and perennial ryegrass, and *Neotyphodium* endophytes, such that the benefits of infection (e.g., insect resistance) but not the disadvantages (i.e., livestock disorders) are conferred (Bouton and Easton 2004). Developing an understanding of the chemical basis of the effects that endophyte infection have on both vertebrates (Siegel and Bush 1996) and invertebrates (Popay and Bonos 2004) and using this information to exploit the diverse

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array of endophytes that occur naturally in tall fescue and ryegrass has been an essential part of this research. Endophytes that have no adverse effects on mammalian health are now commercially available in tall fescue in the United States (Max Q) (Bouton and Easton 2004) and Australasia (Max P) and in ryegrass in New Zealand (AR1) (Fletcher 1999).

Endophyte-mediated insect resistance has been attributed to the presence of alkaloids produced by the fungus in its host. Four major alkaloids, or alkaloid groups, have been identified in *Neotyphodium*-infected grasses: peramine, a pyrrolopyrazine compound (Rowan and Gaynor 1986); lolitrem B and other indole diterpenoids (Gallagher et al. 1981, Munday-Finch et al. 1998); ergot alkaloids, including ergovaline (Garner et al. 1993); and the pyrrolizidine alkaloids *N*-formyl loline and *N*-acetyl loline (Bush et al. 1993). Each of these alkaloids has insect bioactivity, but only in a few cases have endophyte effects on specific insects been linked to the presence of specific alkaloids (Rowan and Gaynor 1986, Siegel et al. 1990, Ball et al. 1997a). In addition, as research has progressed, it has become clear that, beyond these four alkaloid groups, there is a diversity of bioactive metabolites produced by different endophyte strains (Lane et al. 2000, Popay and Bonos 2004).

In developing new endophyte–grass associations for agricultural benefit, each endophyte-mediated effect must be investigated carefully so that the properties of a desirable grass/endophyte association can be better defined. The purpose of our study was to investigate the mechanism of endophyte-mediated resistance to fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), a generalist herbivore of graminoids (Luginbill 1928) and a serious pest of fescue in the United States (Bair et al. 1991), by using a range of different endophytes with an array of alkaloid profiles. This, together with its easy availability and resilience in the laboratory as well as the extensive knowledge concerning its general biology has made it one of the most commonly used insects in endophyte-related studies in the United States. However, since the link between the presence of endophyte and poor performance of *S. frugiperda* was first discovered (Clay et al. 1985, Hardy et al. 1985), little progress has been made in identifying the biochemical causes of resistance to this insect. Moreover reports in the literature provide evidence of endophyte-mediated effects on larval *S. frugiperda* ranging from negative to positive (Popay and Bonos 2004). The study reported in this article was designed to understand better the responses of this lepidopteran to endophyte infection.

Our study evaluated the role played by each of the major known alkaloids in endophyte-mediated resistance against *S. frugiperda.* This was achieved by investigating 1) the effect of different host grass–*Neotyphodium* endophyte combinations producing variable arrays of alkaloids on larval feeding and development and 2) the development of *S. frugiperda* larvae on artificial diets containing individual endophyte-related alkaloids. The effect of a lesser known endophyte, a *Phialophora*-like endophyte, and an endophyte strain that does not produce any of the known alkaloids also were investigated.

Materials and Methods

Source of *S. frugiperda*. The *S. frugiperda* were originally collected around 1971 from alfalfa located near Columbia, MO, and were supplemented with insects collected around 1973 from Bermuda grass near Tifton, GA. Insects from that colony were maintained on a synthetic wheat germ diet (Wilkinson et al. 1972) for >400 generations without additional infusion of feral material. For this study, neonates were placed on the same synthetic wheat germ diet immediately after hatching for 24 h before commencement of feeding trials. This was found to greatly reduce mortality because of handling.

Source of Plant–Endophyte Associations. Three grass species were used—perennial ryegrass; meadow fescue, *Festuca pratensis* Hudson; and tall fescue. Individual host plants of each species were either endophyte-free (E-) or contained an indicated endophyte strain. Infected plants were grown from seed of parent plants artificially inoculated (Latch and Christensen 1985) with selected *Neotyphodium* endophytes. These endophytes were selected because of their commercial promise and/or their differing alkaloid profiles. Tall fescue, meadow fescue, and perennial ryegrass plants naturally infected with their commonly recognized wild-type (WT) endophytes [*N. coenophialum*, *N. uncinatum* (Gams, Petrini, and Schmidt) Glenn, Bacon, and Hanlin, and *N. lolii*, respectively] also were included as were tall fescue plants infected with the *Phialophora*-like endophyte (P+), either alone or in combination with the WT *Neotyphodium* endophyte. All associations used and their known alkaloid profiles are shown in Table 1. 'Grasslands Nui' (GN) and 'Ensign' (EN) were the most commonly used cultivars of perennial ryegrass and meadow fescue, respectively, whereas 'Kentucky 31′ (K31) and 'Johnstone' (JS) were the only two tall fescue cultivars used (Table 1). Johnstone is a hybrid derivative of *L. multiflorum* and *L. perenne* × tall fescue (Buckner et al. 1983). Seeds of all associations listed in Table 1 (with the exception of Predix meadow fescues, which were obtained as mature plants) were sown into peat pots containing potting mix and watered as required. After ≈2 mo, all seedlings were checked for endophyte by staining pseudostem epidermal strips with aniline blue and lactic acid followed by microscopic examination at a magnification of 200×. Endophyte-free plants were rechecked to ensure the endophyte had not been overlooked the first time. Seedlings with the desired endophyte status were then individually transferred into earthenware pots containing potting mix. Plants were maintained outdoors and watered and fertilized on a regular basis. Plants were checked again for endophyte using protein A sandwich-enzyme-linked immunosorbent assay (ELISA) (Reddick and Collins 1988) or the aniline blue stain method just before use in each feeding test.

Table 1. Host plant–endophyte combinations, and their alkaloid profiles, used in *S. frugiperda* feeding tests

Host plant[a] (cultivar[b])	Identifier[c]	*Neotyphodium* taxon[d]	Original host[e]	Alkaloid profile[f]
TF (K31)	WT	*N. coenophialum*	TF	P, EV, L
TF (K31)	AR501	FaTG-3	TF	P, L
TF (K31)	AR502	FaTG-3	TF	P, L
TF (K31)	AR506	FaTG-3	TF	P, L
TF (K31)	AR508	*N. coenophialum*	TF	P
TF (K31)	AR512	*N. coenophialum*	TF	P, L
TF (K31)	AR513	*N. coenophialum*	TF	P, L
TF (K31)	AR514	*N. coenophialum*	TF	P, L
TF (K31)	AR524	*N. coenophialum*	TF	P, L
TF (K31)	AR525	*N. coenophialum*	TF	P, L
TF (K31)	AR542	*N. coenophialum*	TF	P, L[g]
TF (K31)	P+		TF	-
TF (K31)	WT/P+	*N. coenophialum*	TF	P, EV, L
TF (JS)	E+	*N. coenophialum*	TF	P, EV, L
TF (JS)	P+		TF	-
TF (JS)	WT/P+	*N. coenophialum*	TF	P, EV, L
PRG (GN)	WT	*N. lolii*	PRG	P, EV, LB
PRG (GN)	AR17	*Neotyphodium* sp.	PRG	P
PRG (GN)	AR19	*N. lolii*	PRG	P, LB
PRG (GN)	AR20	*N. lolii*	PRG	P, LB
PRG (GN)	AR21	*N. lolii*	PRG	P, LB
PRG (GN)	AR22	*N. lolii*	PRG	P
PRG (GN)	AR23	*N. lolii*	PRG	P, LB
PRG (GN)	AR24	*N. lolii*	PRG	P
PRG (EX.)	AR37	*N. lolii*	PRG	J[f]
PRG (EX.)	AR501	FaTG-3	TF	P, L[h]
MF (EN)	AR29	*N. lolii*	PRG	P, EV, LB
MF (EN)	AR501	FaTG-3	TF	P, L
MF (EN)	AR506	FaTG-3	TF	P, L
MF (EN)	AR512	*N. coenophialum*	TF	P, L
MF (EN)	AR548	*N. coenophialum*	TF	P, EV, L
MF (EN)	AR555	FaTG-2	TF	P, EV, LB
MF (EN)	AR565	*N. coenophialum*	TF	P, EV, L
MF (EN)	AR583	FaTG-2	TF	P, EV
MF (PR)	WT	*N. uncinatum*	MF	L

[a] Plant species in which endophyte was tested. TF, tall fescue; PRG, perennial ryegrass; MF, meadow fescue.
[b] K31, Kentucky 31; JS, Johnstone; GN, Grasslands Nui; EX, experimental line; EN, Ensign; PR, Predix.
[c] Endophyte strain or species identifier. WT, naturally infecting wild-type *Neotyphodium* endophyte for the particular host; P+, *Phialophora*-like endophyte.
[d] Data from Christensen et al. (1993). FaTG-2, *Festuca arundinacea* taxonomic grouping 2; FaTG-3, *F. arundinacea* taxonomic grouping 3.
[e] Host plant species from which endophyte originated.
[f] P, peramine; EV, ergovaline; L, lolines; LB, lolitrem B; J, epoxy janthitrems.
[g] Usual lolines (*N*-formyl and *N*-acetyl) absent, *N*-acetyl norloline present.
[h] *N*-formyl loline only.

Plant–Endophyte Tests. The effect of different endophytes in tall fescue, perennial ryegrass, and meadow fescue on feeding by *S. frugiperda* was determined in choice tests and on growth, development, and survival of larvae in no-choice tests.

Feeding preference of larvae for leaf blades from E− plants compared with blades from infected (E+) plants was determined at three different leaf ages. The terminal-expanding blade of a tiller with a recognizable ligular zone above the sheath was identified as leaf 1, the youngest leaf. The following, next youngest leaf encountered on the tiller was leaf 2, and the next leaf, the oldest, was identified as leaf 3 (Hardy et al. 1986). Plants were trimmed 15 cm above the soil surface immediately before each test. A blade from an E− plant and a blade of equivalent age from an E+ plant were excised as close to the ligular zone as possible, and the cut ends applied to a piece of tape so that the blades lay side by side. The two leaf blades were trimmed to ≈3 to 4 cm and then stuck to the base of a petri dish (15 by 60 mm in diameter) with the tape. A second piece of tape was used to secure the other end of the leaf blades to the dish so that 2–2.5 cm of leaf blade was exposed. A filter paper disc was stuck to the lid of each dish with water to maintain a humid environment. Usually, a minimum of three different host genotypes infected with the same endophyte strain, and three to five E− genotypes, were used in each test, with care taken to evenly distribute plant material from different genotype combinations through all replicates. There were 10 replicate dishes for each leaf age. Seven to 10 1-d-old first instars of *S. frugiperda* were introduced into each dish and placed in total darkness at 26 ± 2°C for 24 h. A rating scale from 0 (no feeding) to 3 (extensive feeding) (Hardy et al. 1985) was used to assess larval feeding damage on each blade. Data were analyzed by analysis of variance (ANOVA) by using GENSTAT 5 (Payne 1987).

Different plant–endophyte associations also were tested for their effects on growth, development, and survival of larvae over 8 d. One-day-old larvae were placed individually into moistened filter paper-lined petri dishes (15 by 60 mm in diameter) containing leaf blade material from either E− or E+ plants ($n = 20$ dishes). Leaf blade material of mixed age and from at least three different host genotypes infected with the same endophyte strain was combined. Dishes were held under a photoperiod of 16:8 (L:D) h at 22–26 ± 2°C (the temperature at which a particular bioassay was conducted was dependent upon the availability of incubators; hence, the range of temperatures used). Fresh leaves were provided, and old leaves removed, every other day. Water was added to the filter paper as required. Instar and survival were assessed every day, and larvae were weighed after 5 and 8 d. Instar and survival data are only presented for days 5 and 8 in the results. Experiments were not continued after 8 d as herbage was a limiting factor in many cases. Larval weights were log transformed as necessary to normalize the data and analyzed by ANOVA. Instar data also were analyzed by ANOVA and survival data by the Fisher exact test. The small differences in temperature at which bioassays were conducted had a large effect on growth and development of larvae. Because of this, weight and instar data are expressed as percentages of the mean weight and instar of larvae reared on appropriate endophyte-free controls. A pooled SEM for each grass species is presented in each table.

Alkaloid Analyses. Herbage samples were taken (from ground level to 15 cm) from all test plants at the time of each bioassay. These were immediately frozen, freeze-dried, ground to a fine powder, and stored frozen until chemical analysis. Grass samples were analyzed for peramine, lolitrem B, and ergovaline by using minor variations of previously published high-

Table 2. Tall fescue: mean feeding scores (pooled SED = 0.294) of larval *S. frugiperda* in a feeding choice test between endophyte-infected and endophyte-free leaf blades, and weight, instar, and survival of larvae in a no-choice test

Identifier[a]	Feeding score[b]		Wt (%)[c]		Instar (%)[c]		Survival (%)	
	E+	E−	Day 5	Day 8	Day 5	Day 8	Day 5	Day 8
K 31 E−			100	100	100	100	95	95
K 31 AR501	2.5**	1.6	135	125	104	100	95	95
K 31 AR502	2.4**	1.5	103	112	102	100	100	100
K 31 AR506	2.1	1.8	136*	123	103	100	95	95
K 31 AR508	1.2***	2.5	127	137*	103	100	100	90
K31 AR512	1.2**	2.1	88	87	97	101	100	100
K31 AR513	1.1**	2.0	75	70*	89	100	100	100
K31 AR514	1.2**	2.0	156**	132*	106	101	100	100
K31 AR524	1.7	1.5	142*	135*	100	101	100	100
K31 AR525	1.5	1.8	94	96	99	100	100	100
K31 AR542	2.3	1.7	126	131*	100	100	100	100
K31 WT	1.3**	2.1	139*	149**	101	101	90	90
K31 P+	1.5	2.1	129	109	101	100	95	95
K31 WT/P+	1.4	1.9	90	89	96	101	95	95
Pooled SEM			13.0	10.8	2.3	0.6		
JS E-−			100	100	100	100	100	100
JS WT	1.6	2.2	88	83	104	100	95	95
JS P+	2.2	2.0	98	77*	100	99	95	95
JS WT/P+	1.6*	2.3	88	71**	96	100	95	95
Pooled SEM			8.3	6.9	3.2	1.2		

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ANOVA for within cultivar comparisons.

[a] Cultivar of tall fescue: K31, Kentucky 31; JS, Johnstone. AR, endophyte strain or species identifier; WT, naturally infecting wild-type *Neotyphodium* endophyte; P+, *Phialophora*-like endophyte.

[b] Scoring after 24-h feeding (averaged over three leaf ages; $n = 30$) by using a system based on Hardy et al. (1985), where 0, no feeding and 3, extensive feeding.

[c] Weight and instar data for each endophyte treatment ($n = 20$) obtained after 5 and 8 d and expressed as percentages of endophyte-free values (assigned a value of 100%).

performance liquid chromatography (HPLC) methods (Barker et al. 1993). Lolines were analyzed by gas chromatography (GC) using a modification of the method of Yates et al. (1990). Grass samples (100 mg) were extracted for lolines in 1 ml of dichloroethane (containing 4-phenylmorpholine as internal standard) supplemented with 50 μl of a 5% NH_3/40% MeOH solution. Four glass beads (≈5 mm in diameter) were added to each vial to aid in the extraction process. After vigorous mechanical disruption for 40 s, samples were extracted for 60 min on a rotating arm (21 rpm). After filtration, the resulting extracts were analyzed by GC.

Artificial Diet Tests. Development of *S. frugiperda* larvae on artificial diets containing representatives of the four known groups of endophyte-related alkaloids was investigated. These were lolitrem B (indole-diterpenoid), peramine (pyrollopyrazine), *N*-acetyl loline, *N*-formyl loline (pyrrolizidine), ergovaline, ergocryptine and lysergic acid amide (ergot alkaloids). Each alkaloid was tested at several concentrations. A wheat germ artificial diet (Wilkinson et al. 1972) was used. Alkaloids dissolved in solvent (water for peramine and ethanol for the remainder) were incorporated into the diet at the lowest practical temperature (≈52–55°C) in an attempt to minimize chemical degradation. The amount of alkaloid added was calculated on a dry weight of diet basis. Solvent alone was added to the control diets. One-day-old larvae were placed individually into petri dishes containing either control diet or alkaloid-treated diet ($n = 20$ dishes). A moistened filter paper disc was attached to the lid of each dish and watered as required to maintain humidity. Petri dishes were held under a 16:8 (L:D) photoperiod at 22–26 ± 2°C. Larval weights were recorded on days 5 and 8 after which the experiments were terminated. Data were analyzed using a Student *t*-test

Results

Plant–Endophyte Tests. Leaf age had no effect on larval damage for any of the cultivar–endophyte associations, and so damage scores were averaged over the three leaf ages. A standard error of the difference (SED) within each grass species, pooled across the three leaf ages and the different endophyte strains, is presented in each table.

Infection of K31 tall fescue with WT *N. coenophialum* alone and with endophytes AR508, AR512, AR513, and AR514 significantly reduced feeding scores in preference tests (Table 2). Despite this, AR513 was the only one of these strains to significantly reduce larval weight in K31 tall fescue no-choice tests, whereas larvae fed WT, AR508, or AR514 had higher weight gains than larvae fed E− leaf blades. The remaining *Neotyphodium* strains either had no effect on larval feeding (AR506, AR524, AR525, and AR542), or significantly increased it (AR501 and AR502). Mean weight of larvae fed AR524, AR506, and AR542 was higher on one or both assessment days, relative to larvae fed E− leaf blades. None of these strains significantly affected development or survival of the larvae. Infection with the *Phialophora*-like endophyte, either alone (P+) or in combination with *N. coenophialum* (WT/P+), had no effect on feeding or on growth and development.

Table 3. Perennial ryegrass: mean feeding scores (pooled SED = 0.261) of larval *S. frugiperda* in a feeding choice test between endophyte-infected and endophyte-free leaf blades, and weight, instar and survival of larvae in a no-choice test

Identifier[a]	Feeding score[b]		Wt (%)[c]		Instar (%)[c]		Survival (%)	
	E+	E−	Day 5	Day 8	Day 5	Day 8	Day 5	Day 8
GN E−			100	100	100	100	95	90
GN AR17	1.4***	2.4	24.6***	15.2***	75***	77***	100	90
GN AR19	2.5	2.2	85.1	90.9	100	97	100	100
GN AR20	2.0	2.3	107	99	100	98	100	100
GN AR21	2.3	2.1	84	94	98	96	95	95
GN AR22	0.8***	2.6	41***	41***	90**	88***	95	95
GN AR23	2.3	2.3	62*	69*	100	92*	100	100
GN AR24	1.8	2.3	79	85	98	93*	90	90
GN WT			39***	28***	83***	82***	95	95
Pooled SEM			7.7	6.7	2.2	2.3		
EX E−			100	100	100	100	85	80
EX AR37	0.7***	2.5	16***	15***	76***	96	80	75
EX AR501	1.6**	2.4	65	89	99	100	95	90
Pooled SEM			10.4	7.1	1.9	1.5		

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ANOVA for within cultivar comparisons.

[a] Perennial ryegrass cultivar: GN, Grasslands Nui; EX, experimental. AR, endophyte strain or species identifier; WT, *N. lolii* wild-type; E−, endophyte free.

[b] Scoring after 24-h feeding (averaged over three leaf ages; $n = 30$) by using a system based on Hardy et al. (1985) where 0, no feeding and 3, extensive feeding.

[c] Weight and instar data for each endophyte treatment ($n = 20$) obtained after 5 and 8 d and expressed as percentages of endophyte-free values (assigned a value of 100%).

In JS tall fescue, feeding scores were significantly higher on leaf blades from E− plants than on leaf blades from WT/P+ plants but not different from plants infected with either endophyte alone (Table 2). In the no-choice tests, significant weight reductions were measured for larvae reared for 8 d on WT/P+ and P+ leaf blades.

Perennial ryegrass leaf blades infected with AR17, AR22, AR37, and AR501 suffered significantly less feeding damage than E− blades in choice tests (Table 3). There were no differences in feeding between E− perennial ryegrass blades and blades infected with AR19, AR20, AR21, AR23, and AR24. Larvae reared on perennial ryegrass infected with strains AR17, AR22, AR23, AR37, or WT *N. lolii,* weighed significantly less on days 5 and 8 than larvae fed E− ryegrass (Table 3). This effect was particularly strong in AR17 and AR37 associations with larvae weighing less than one-quarter to one-sixth of those reared on E− ryegrass. The smallest significant weight reduction of approximately one-third was observed in larvae fed ryegrass infected with strain AR23, although this strain had not reduced larval feeding in the choice test. Weight reductions of larvae were generally accompanied by delays in molting leading to significant instar differences but not by reduced larval survival. Larval weight, instar, and survival were not affected by infection of perennial ryegrass with strains AR19, AR20, AR21, and AR501. AR24 also had little effect, although the mean larval instar on day 8 was slightly lower than for larvae fed E− ryegrass.

All endophyte strains tested in meadow fescue significantly reduced feeding and weight gain by *S. frugiperda* compared with E− meadow fescue (Table 4). Reductions in weight gains in the no-choice tests for larvae fed meadow fescue with AR29, AR512, AR555, and AR583 were such that mean larval weights were <10% of the mean weight of control larvae. Development (instar) was also significantly delayed by these endophyte strains, whereas AR555 also significantly reduced larval survival. The smallest difference in feeding scores between E+ and E− blades of meadow fescue in the choice tests was recorded for the naturally infecting *N. uncinatum* WT strain in Predix. Larvae reared on PRWT, and on ENAR548 and AR565, also had relatively small weight reductions and little or no effect on their development compared with the effect of other endophytes.

Alkaloid Analysis of Herbage. All associations were true to type concerning presence or absence of each alkaloid (Tables 5–7). However, alkaloid concentrations were highly variable between associations containing different endophyte strains.

Artificial Diet Tests. For reasons described above, the weight of larvae reared on alkaloid-treated diets is expressed as a percentage of the weight of control larvae. Incorporation of lolitrem B, *N*-acetyl loline, *N*-formyl loline, and ergovaline individually into artificial diet had no effect on larval weight on either assessment day at any concentration tested (Table 8). Peramine also had no effect at 10, 30, or 100 μg/g but significantly reduced mean larval weight at 50 μg/g on day 5, but not on day 8. In a retest, peramine had no effect on larval mass at 50 μg/g. Lysergic acid amide at 10 μg/g significantly increased larval mass on day 5 but not on day 8. In a repeat test at this concentration, larval mass was significantly ($P < 0.05$) higher on both assessment days. Larval weights were not significantly different to the weight of control larvae at any other concentration of lysergic acid amide. Larvae reared on diet amended with ergocryptine at 50 μg/g weighed significantly more than those fed control diet on day 8 (but not on day 5). On repeating the test, a different result was obtained where treatment and control lar-

Table 4. Meadow fescue: mean feeding scores (pooled SED = 0.151) of larval *S. frugiperda* in a feeding choice test between endophyte-infected and endophyte-free leaf blades, and weight, instar, and survival of larvae in a no-choice test

Identifier[a]	Feeding score[b]		Wt (%)[c]		Instar (%)[c]		Survival (%)	
	E+	E−	Day 5	Day 8	Day 5	Day 8	Day 5	Day 8
EN E−			100	100	100	100	95	95
EN AR29	0.4***	2.9	9***	4***	56***	75***	100	100
EN AR501	0.5***	3.0	28***	20***	76***	93**	100	100
EN AR506	0.6***	2.9	17***	14***	70***	89***	95	90
EN AR512	0.2***	3.0	7***	3***	59***	71***	80	70
EN AR548	0.8***	3.0	56**	75	94	100	100	100
EN AR555	0.2***	3.0	5***	5***	56***	78***	60*	45**
EN AR565	0.8***	2.9	41***	53***	88***	100	90	90
EN AR583	0.1***	3.0	4***	1***	54***	67***	90	70
Pooled SEM			3.5	3.5	2.4	2.0		
PR E−			100	100	100	100	100	100
PR WT	1.7***	2.5	69**	75*	96	105	95	95
Pooled SEM			7.9	8.2	3.5	2.7		

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ANOVA for within cultivar comparisons.

[a] Meadow fescue cultivar: EN, Ensign; PR, Predix. AR, endophyte strain or species identifier; WT, *N. uncinatum* wild-type; E−, endophyte-free.

[b] Scoring after 24-h feeding (averaged over three leaf ages; $n = 30$) by using a system based on Hardy et al. (1985) where 0, no feeding and 3, extensive feeding.

[c] Weight and instar data for each endophyte treatment ($n = 20$) obtained after 5 and 8 d and expressed as percentages of endophyte-free values (assigned a value of 100%).

val weights were similar on both days. There were no significant larval weight differences at any other concentration of ergocryptine.

Discussion

The effects of *Neotyphodium* endophyte infection on *S. frugiperda* were extremely variable within each of the three hosts and highly dependent upon the species or strain of endophyte present. In addition these results suggest that host species is also extremely important in regulating endophyte effects on *S. frugiperda* with endophyte-induced resistance to this insect weakest in tall fescue, strongest in meadow fescue, and intermediate in perennial ryegrass.

In the tall fescues, larval feeding was reduced, relative to E− grass, by some associations, whereas in other associations, the reverse was true (Table 2). Deterrency in the preference test did not equate with lower weight gains in a no-choice test, however, with only one association having a negative effect on *S. frugiperda* weight gain, whereas five associations had a positive effect. Other tall fescue associations had no effect on either feeding or development. In

Table 5. Mean concentrations (micrograms per gram) of the four major alkaloids or alkaloid groups for all tall fescue plants used in choice and no-choice tests

Identifier[a]	Choice test				No-choice test			
	P[b]	EV[b]	LB[b]	L[c]	P[b]	EV[b]	LB[b]	L[c]
KY AR501	3.4	0.0	0.0	175.7	4.5	0.0	0.0	244.7
KY AR502		No sample				No sample		
KY AR506	8.5	0.0	0.0	348.4	8.5	0.0	0.0	348.4
KY AR508	9.5	0.0	0.0	0.0	10.7	0.0	0.0	0.0
KY AR512	7.2	0.0	0.0	865.0+	7.3	0.0	0.0	892.8
KY AR513	8.6	0.0	0.0	1,982.3+	9.1	0.0	0.0	1,898.0+
KY AR514	4.6	0.0	0.0	778.6	5.1	0.0	0.0	833.8
KY AR524	4.7	0.0	0.0	556.1++	4.7	0.0	0.0	556.1++
KY AR525	5.7	0.0	0.0	664.9	5.4	0.0	0.0	587.6
KY AR542	7.3	0.0	0.0	0.0++	6.3	0.0	0.0	0.0++
KY WT	4.3	1.0	0.0	444.6	5.0	0.5	0.0	1,393.4+
KY P+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KY WT/P+	2.4	0.8	0.0	264.1	3.0	0.3	0.0	797.2+
JS WT	4.4	0.4	0.0	1,471.5+	5.0	0.5	0.0	1,668.0+
JS P+	0.0	0.0	0.0	0.0	0.1[d]	0.0	0.0	0.0
JS WT/P+	3.2	0.2	0.0	863.7+	2.8	0.3	0.0	872.2+

[a] Tall fescue cultivar: KY, Kentucky 31; JS, Johnstone. AR, endophyte strain or species identifier; E+, naturally infecting wild-type *Neotyphodium coenophialum;* P+, *Phialophora*-like endophyte.

[b] P, peramine; EV, ergovaline; LB, lolitrem B.

[c] Values for the loline alkaloids (L) are the sum of the concentrations of *N*-acetyl and *N*-formyl lolines. The presence of *N*-acetyl norloline is indicated by a +. A standard for this compound was not available, thus concentrations were estimated using the parameters for *N*-acetyl loline. +, 30–100 μ g/g; ++, 101–300 μ g/g.

[d] Detection uncertain, trace quantities if present.

Table 6. Mean concentrations (micrograms per gram) of the four major alkaloids or alkaloid groups for all perennial ryegrass plants used in choice and no-choice tests

Identifier[a]	Choice test				No-choice test			
	P[b]	EV[b]	LB[b]	L[b]	P[b]	EV[b]	LB[b]	L[b]
GN AR17	0.6	0.0	0.0	0.0	0.6	0.0	0.0	0.0
GN AR19	2.6	0.0	1.1	0.0	2.5	0.0	1.1	0.0
GN AR20	3.5	0.0	1.3	0.0	2.9	0.0	1.1	0.0
GN AR21	2.2	0.0	1.1	0.0	1.9	0.0	0.9	0.0
GN AR22	34.9	0.0	0.2[c]	0.0	32.5	0.0	0.2[c]	0.0
GN AR23	4.1	0.0	1.7	0.0	3.9	0.0	1.5	0.0
GN AR24	20.2	0.0	0.0	0.0	18.4	0.0	0.0	0.0
GN WT		No bioassay			21.2	0.2	8.0	0.0
EX AR37	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EX AR501	17.7	0.0	0.0	131.0[d]	16.5	0.0	0.0	128.7[d]

[a] Perennial ryegrass cultivar: GN, Grasslands Nui; EX, experimental. AR, endophyte strain or species identifier; WT, naturally infecting wild-type *Neotyphodium lolii.*
[b] P, peramine; EV, ergovaline; LB, lolitrem B; L, lolines.
[c] Detection uncertain, trace quantities if present.
[d] Only *N*-formyl loline present.

contrast to these results, all endophytes in meadow fescue adversely affected larval feeding and growth of *S. frugiperda,* although again potency was highly strain dependent (Table 4). Extreme effects were observed with several endophytes (AR29, AR512, AR555, and AR583) in this host. In most cases, development also was delayed, whereas survival was significantly reduced with one association (AR555). In the case of perennial ryegrass, the effects of endophyte infection on feeding and development of *S. frugiperda* larvae ranged from none to potently adverse (Table 3).

Interhost comparisons with strains AR501, AR506, and AR512 support the notion that *Neotyphodium* endophytes generally have stronger activity against *S. frugiperda* when present in meadow fescue, less activity when in perennial ryegrass, and less still in tall fescue (Tables 2–4). The causes of such differences are uncertain, but they may relate to the finding that meadow fescue supports greater quantities of the same endophyte than perennial ryegrass, which in turn supports more than tall fescue (Christensen et al. 1997). Quantitative ELISA determinations of endophyte content in the grass samples containing AR501, AR506, and AR512 used in our study fully support this view (unpublished data). Interestingly, Breen (1993) found limited evidence of a link between endophyte content (as measured by hyphal counts) and resistance to *S. frugiperda.* Concentrations of the alkaloids, which in some cases seem to be related to the amount of endophyte (Ball et al. 1995), also were generally higher in meadow fescue than in perennial ryegrass or tall fescue in our study (Tables 5–7). Such differences have previously been reported for a set of the plants corresponding to those used in this study which were grown under the same conditions and harvested at the same time (Ball and Tapper 1999).

Most of the previous work concerning the interaction between *S. frugiperda* and *Neotyphodium* endophytes has centered upon natural associations (equivalent to the WT treatments in this study) of tall fescue and perennial ryegrass. As in our study, Braman et al. (2002) found that perennial ryegrass infected with WT *N. lolii* had a greater effect on growth and development of *S. frugiperda* than tall fescue infected with WT *N. coenophialum.* Other studies with perennial ryegrass also have consistently shown moderate-to-

Table 7. Mean concentrations (micrograms per gram) of the four major alkaloids or alkaloid groups for all meadow fescue plants used in choice and no-choice tests

Identifier[a]	Choice test				No-choice test			
	P[b]	EV[b]	LB[b]	L[c]	P[b]	EV[b]	LB[b]	L[c]
EN AR29	27.9	0.1	1.7	0.0	23.7	0.0	1.4	0.0
EN AR501	41.7	0.0	0.0	1041.0	38.9	0.0	0.0	1,950.4+
EN AR506	29.3	0.0	0.0	1945.6+	30.9	0.0	0.0	2,195.4+
EN AR512	28.3	0.0	0.0	781.7+	18.5	0.0	0.0	1,151.3+
EN AR548	23.3	4.9	0.0	4,024.5+++	19.1	4.2	0.0	3,224.0+++
EN AR555	17.2	8.0	0.7	0.0	8.6	4.2	0.5	0.0
EN AR565	6.3	3.7	0.0	1,807.6++	5.6	7.4	0.0	2,571.4++
EN AR583	12.4	5.4	0.0	0.0	7.0	2.7	0.0	0.0
PR WT	0.3[d]	0.0	0.0	947.0++	0.0	0.0	0.0	1,237.0++

[a] Meadow fescue cultivar: EN, Ensign; PR, Predix. AR, endophyte strain or species identifier; WT, naturally infecting wild-type *N. uncinatum.*
[b] P, peramine; EV, ergovaline; LB, lolitrem B.
[c] Values for the loline alkaloids (L) are the sum of the concentrations of *N*-acetyl and *N*-formyl lolines. The presence of *N*-acetyl norloline is indicated by a +. A standard for this compound was not available, thus concentrations were estimated using the parameters for *N*-acetyl loline. +, 30–100 μ g/g; ++, 101–300 μ g/g; +++, >300 μ g/g.
[d] Detection uncertain, trace quantities if present.

Table 8. Mean weights of larvae confined to artificial diet containing different alkaloids at varying concentrations after 5 and 8 d

Alkaloid	Concn (μg/g)	Larval wt (%)	
		Day 5	Day 8
Lolitrem B	1.0	93.2	89.7
	10.0	114.5	101.5
	20.0	125.9	122.5
	Pooled SEM	13.4	8.6
Peramine	10.0	98.5	114.2
	30.0	93.5	88.9
	50.0	69.5*	78.7
	100.0	100.1	103.7
	Pooled SEM	8.5	6.6
N-Acetyl loline	500.0	106.3	111.9
	1000.0	106.1	114.9
	Pooled SEM	8.4	11.3
N-Formyl loline	500.0	85.9	102.8
	1000.0	98.6	98.7
	Pooled SEM	8.6	11.8
Lysergic acid amide	1.0	112.4	104.1
	10.0	146.3**	129.7
	20.0	119.7	112.3
	50.0	109.4	102.1
	100.0	111.0	112.0
	Pooled SEM	10.2	8.6
Ergovaline	1.0	101.5	95.3
	10.0	91.5	100.1
	20.0	89.8	100.1
	Pooled SEM	7.6	7.6
Ergocryptine	1.0	81.0	83.5
	10.0	100.6	100.2
	20.0	84.0	80.8
	50.0	124.4	139.4*
	100.0	93.0	107.0
	Pooled SEM	11.7	12.3

Weights are expressed as percentages of the weight of larvae maintained on control (no alkaloid added) diet.

*, $P < 0.05$; **, $P < 0.01$, Student *t*-test.

strong deleterious effects of WT *N. lolii* infection on feeding by neonates (Hardy et al. 1985, Clay et al. 1993) and mass of larvae reared from first instars (Clay et al. 1985, 1993; Hardy et al. 1985; Bultman and Ganey 1995). Survival, total developmental time, and pupal mass also can be adversely affected by WT *N. lolii* infection of ryegrass, although these effects are not always apparent (Clay et al. 1985, 1993; Hardy et al. 1985; Bultman and Ganey 1995). The large reductions in the mass of larvae reared on WT perennial ryegrass compared with larvae fed E− ryegrass on days 5 and 8, and the associated lag in instar observed in our study (Table 3), corroborate these previous findings.

Compared with other host grasses, results with tall fescue have been more variable. Early work indicated that neonate feeding and development were adversely affected by WT *N. coenophialum* infection of tall fescue (Clay et al. 1985, Hardy et al. 1986). Later, Clay et al. (1993) recorded a large decline in survival of *S. frugiperda* maintained on WT-infected tall fescue but observed no feeding deterrence. Other studies have failed to show consistent protective effects against *S. frugiperda* of WT *N. coenophialum* infection in tall fescue. Breen (1993) and Davidson and Potter (1995) found no effect of *N. coenophialum* on feeding behavior and development of third instars, although there is much evidence to suggest that older instars of *S. frugiperda* and other insects are less sensitive to the effects of endophytes than early instars (Hardy et al. 1986, Kindler et al. 1991, Potter et al. 1992, McDonald et al. 1993). Bultman and Conard (1997), however, found that endophyte infection increased 8-d larval mass and reduced developmental time of *S. frugiperda* reared from neonates but reduced pupal mass. A similar increase in growth rate of third to fifth instars feeding on WT tall fescue was found by Bultman and Bell (2003). In the current study, first instars feeding on K31 leaf blades infected with WT were deterred in the feeding choice test, but larvae fed the same association in the no-choice test weighed substantially more than control larvae fed E− grass after 5 or 8 d (Table 2). However, there were no effects at all on larvae fed JS tall fescue, a hybrid derivative, infected with WT *N. coenophialum* alone. Thus, our results with tall fescue are also inconclusive. In addition, we did not find that leaf age had any bearing on the level of deterrence. This is contrary to a previous report, which found that as leaf age increased neonate preference for E− over E+ leaves also increased (Hardy et al. 1986). Similarly, Braman et al. (2002) noted that deleterious effects of endophyte on *S. frugiperda* larvae became more apparent as plants aged. A number of factors may explain this discrepancy, among which are differences in *S. frugiperda* strain, host plant status, and the interaction between the endophyte and plant.

Overall, our results suggest that endophyte-enhanced resistance in tall fescue against *S. frugiperda* is a comparatively weak phenomenon and is easily masked by other parameters. Thus, it is not possible to conclude that the small but significant reduction in weight gain of larvae reared on JS infected with the P± endophyte alone (Table 2) was due to the presence of this endophyte. Nevertheless, our results with tall fescue reveal two unusual phenomena that merit closer examination. First, the contrasting effects of some endophytes (e.g., AR508, AR514, and WT) on larvae in the choice and no-choice tests where they were deterred from feeding in the former but showed greater weight gains in the latter was unexpected (Table 2). Greater weight gains also were observed with other endophytes (e.g., AR506, AR524, and AR542) where larvae had shown no feeding preference in the choice tests. Furthermore, repeated testing sometimes revealed that, even within a matter of days, larval preferences could change drastically (e.g., from deterrence of a particular strain to "attraction") even when identical plants were used (unpublished data). This also was seen with tall fescue infected with the P± endophyte alone. The enhancement of larval performance on E+ plants is the second unusual phenomenon. Repeated testing using only some of the associations (unpublished data) gave the same result. Interestingly, there are precedents in the literature where performance of *S. frugiperda* and *Spodoptera eridania* (Cramer) larvae has apparently improved on E+ treatments (Clay 1991, Breen 1993, Bultman and Conard 1997, Bultman and Bell 2003). Similarly, positive effects of endophyte infection on insect fitness and performance have been recorded for other species such as redlegged grasshopper, *Melanoplus femurrubrum* (De Geer) (Lopez et al.1995) and the aphid *Aploneura lentisci* (Passerini) (Popay et al. 2004).

The outcome of any encounter between an insect and its host plant depends on a balance between those factors that have a negative effect on the insect such as feeding deterrents or toxins and those that have a positive effect such as feeding stimulants and nutrients. The presence of endophyte-produced alkaloids in the plant is usually associated with feeding deterrence, toxicity, or both, depending on the sensitivity of the particular insect to the presence of bioactive compounds and the concentration and distribution of these compounds in relation to the insect's feeding sites. The effect of alkaloids on feeding will be further moderated by the hunger of the insect. Thus, *S. frugiperda* confined to E+ tall fescue may be driven by hunger to feed on it, even though it is less preferred than E− plants in choice tests, or, alternatively, may become habituated or even attracted to the endophyte-produced compounds. Under these circumstances, and provided there is no toxic effect of the alkaloids, then growth and development of the insect would be expected to at least match that of the insects on an E− diet. That larval performance on E+ tall fescue exceeded that on E− in some cases suggests there may be other factors relating to host plant quality that influence *S. frugiperda* larvae.

The inconsistent and changeable nature of the feeding test results also may be driven by environmental interactions with the plant and the endophyte causing fluctuations in the alkaloid content of the plant. Concentrations of the major alkaloids in E+ grasses vary considerably over time (Rottinghaus et al. 1991, Bush et al. 1993, Ball et al. 1995); therefore, the time of year in which the tests were carried out (all bioassays were conducted in spring and summer 1996) may have had an effect. No apparent trends in the concentrations of the various major alkaloids were observed, however, that could explain the variable responses (Table 8), but it should be noted that the alkaloid content of the plants was determined on plant material harvested at ground level (and therefore included leaf sheath) whereas the larvae were fed exclusively on leaf blades. Concentration of the known major alkaloids, except peramine, is generally greater in the leaf sheath than the leaf blade in vegetative plants (Siegel and Bush 1996; Ball et al. 1997b,c; Justus et al. 1997; Lane et al. 2000). In addition, in planta distribution of alkaloids is a function of plant genotype, leaf age, and tissue type (Spiering et al. 2005). Thus, to establish a relationship between insect response and alkaloids, the plant material that best represents that which the insect is fed needs to be analyzed. Regardless, it could be expected that the alkaloid spectra of the different endophytes tested in this study would give some clues as to the compounds most likely to be affecting *S. frugiperda* larvae. Yet, none of the major known alkaloids were categorically implicated in endophyte-mediated resistance against this insect. The presence of moderate-to-high concentrations of all four alkaloid groups in susceptible or only mildly effective associations (namely, lolitrem B [e.g., AR19-21 in ryegrass], lolines [WT in tall fescue and meadow fescue], ergovaline [AR548 in meadow fescue], and peramine [AR24 in ryegrass)] (Tables 2–7) provides strong evidence of their inactivity. Absence of a particular alkaloid in a resistant association also may indicate inactivity of that alkaloid but cannot necessarily be interpreted as such because *S. frugiperda* may be sensitive to more than one alkaloid. Resistance was strong in some associations lacking specific alkaloids: lolitrem B (e.g., AR17 and AR37 in ryegrass and AR512 and AR583 in meadow fescue), lolines (AR17, AR37, and WT in ryegrass and AR29 and AR555 in meadow fescue), ergovaline (AR17 and AR37 in ryegrass and AR506 and AR512 in meadow fescue), and peramine (AR37 in ryegrass) (Tables 1–4).

The results of our artificial diet study provide further evidence that none of the known major alkaloids affect *S. frugiperda* larvae. No consistent deleterious effects of lolitrem B, peramine, *N*-acetyl loline, *N*-formyl loline, lysergic acid amide, ergovaline, or ergocryptine on *S. frugiperda* development were observed (Table 8). For peramine, although the initial bioassay indicated significant activity at 50 μg/g, a repeat test at the same concentration and a further test at 100 μg/g failed to detect any effect. Previous studies investigating the effect of some of the major alkaloids on *S. frugiperda* are also inconclusive. Hardy et al. (1986) found that neonate feeding patterns were not related to loline concentrations in tall fescue leaves of different ages, whereas Riedell et al. (1991) found, in artificial diet bioassays, that many naturally occurring and synthetic lolines deterred feeding by *S. frugiperda* larvae, adversely affected their development at 2000 μg/g, or both. However, it is uncertain whether Riedell et al. (1991) based the rate at which lolines were added on the dry weight or wet weight of the diet. Working on ergot alkaloids, Clay and Cheplick (1989) found that they could function as antibiotics (ergonovine), feeding deterrents (ergocryptine and elymoclavine), or both (ergotamine and agroclavine) against *S. frugiperda* larvae. These authors concluded, however, that ergot alkaloids played only a minor role *in planta* as antifeedant effects were only seen at concentrations well in excess of those usually encountered in endophyte-infected tall fescue.

The strong resistance elicited in perennial ryegrass by strain AR37, an association seemingly free of all of the major alkaloids, raises the possibility of there being other, as yet unidentified compounds, with insecticidal properties that are produced by endophytes. A similar effect of AR37 has been observed with other insect pests (Ball et al. 1994, Popay and Wyatt 1995, Jensen and Popay 2004, Popay et al. 2004, Pennell et al. 2005). Unlike other known endophytes, AR37 produces epoxy-janthitrems (Tapper and Lane 2004), although these alkaloids have yet to be tested directly for their bioactivity against insects. Minor known alkaloids that are closely related to the major alkaloids but are not screened for routinely (Shelby et al. 1997, Munday-Finch et al. 1998) also may have a role. For example, artificial diet containing paxilline, an indole diterpenoid found in WT *N. lolii*-infected perennial ryegrass (Weedon and Mantle 1987), has been reported to reduce development and survival of *S. frugiperda* larvae (Dowd et al. 1988). Whatever the answer, our results illustrate the complexity of insect response to endophyte infection and the need for further research in this area. They also show that in the absence of known mammalian toxins, such as lolitrem B and ergovaline, endophytes can be used to provide their host plants with resistance against insect pests such as *S. frugiperda.*

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